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PRINCIPAL INVESTIGATOR: Daniel J. Olson, Ph.D.

CONTRACTING ORGANIZATION: Pennsylvania State University
Hershey, Pennsylvania 17033

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Daniel J. Olson, Ph.D.

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Pennsylvania State University
Hershey, Pennsylvania 17033

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The implications for determining whether *wnt*-5a and *wnt*-4 act as mediators of normal cell growth are relevant to the detection, diagnosis, and treatment of breast cancer. The intent of this proposal has been to develop new mammary epithelial cell lines which stably express antisense *wnt*-5a or *wnt*-4 in order to determine whether the loss of endogenous gene expression of either is important for cell transformation to occur. It was found that disruption of *wnt*-5a leads to cell transformation and ectopic *wnt*-5a gene expression in transformed cells reverts cells to a more differentiated phenotype. This has been tested in two different human malignant cell lines where overexpression of *wnt*-5a in cells which have lost the normal expression of *wnt*-5a reverts transformation and tumorigenesis. This has important ramifications for breast cancer in which loss of heterozygosity of chromosome 3p (where *wnt*-5a has been mapped) correlates with a poor prognostic outcome. It may be that *wnt*-5a is a previously undescribed anti-oncogene the loss of which is important in the multi-step progression of cellular events that lead to cancer growth.

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FOREWORD

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Daniel Allison 7/30/97

PI - Signature Date

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INTRODUCTION

Wnt-genes

The*Wnt* family of genes express highly conserved, growth factor-like molecules some of which are thought to be implicated in mammary tumorigenesis (1). *Wnt*-proteins contain 350-380 amino acids, including 24 highly conserved cysteines and a several glycosylation sites which tend to be poorly conserved (2). The amino terminal amino acids are extremely hydrophobic and encode a signal peptide sequence which is cleaved at a potential signal peptidase cleavage site (2, 3). When in the cell, the protein is bound to a chaperonin-binding protein and is associated with the endoplasmic reticulum and Golgi apparatus (4). *Wnt* proteins enter the secretory pathway and are tightly bound to the cell surface and/or extracellular matrix (5-9), acting autocrinely or paracrinely (10). The biologic role of *wnt*-genes in embryonic and adult tissues involves tissue organization and patterning (3, 11-18). The mechanism of action of *wnt*-genes is still not clear but important clues have recently been reported. That is, *wnt*-genes interact with several signalling pathways including those involved in cell adhesion (19-21), gap junctional communication (22, 23), the adenomatous polyposis coli (APC) tumor suppressor gene (24), and telomerase enzyme activity (25).

Wnt-genes in cell transformation and tumorigenesis

The *wnt*-1 gene was first identified in mouse mammary tumor virus (MMTV) induced tumors occurring often in certain mouse strains (26). Analysis of these tumors revealed that MMTV proviral integration does not interrupt the protein coding sequences leaving an intact protein (27). Therefore, inappropriate expression of the normal *wnt*-1 protein is implicated in cellular transformation. *Wnt*-2 and *Wnt*-3 have also been isolated from several mouse mammary tumors with activated MMTV provirus (28, 29). Furthermore, an *in vivo* role for MMTV directed *wnt*-1 expression in mammary tumorigenesis has been directly shown in transgenic mice (30, 31). Importantly, those *wnt*-genes which are associated with growth deregulation are mainly those which are absent in normal adult breast, suggesting that aberrant *wnt*-gene expression can contribute to the development of mouse mammary cancers (1, 32). In cell culture, certain *wnt*-gene family members are able to transform the cell phenotype (33-36). It is interesting that the extent of *wnt*-mediated transformation depends on the specific *wnt* gene being ectopically expressed (37).

It is not known how some *wnt*-gene family members are involved in cell transformation and tumorigenesis, but as suggested above likely involves disruption of normal spatio-temporal *wnt* gene expression. In support of this, Olson and Papkoff (1994) previously found that *wnt*-5a endogenously expressed in C57MG mouse mammary epithelial cells may control normal cell growth. That is, loss of normal gene expression of *wnt*-5a and *wnt*-4 in the presence of ectopically expressed *wnt*-1, *wnt*-2, or *neu* T (c-erbB-2) correlates with cell transformation (38). Hepatocyte growth factor (HPG), a poor prognostic indicator in some cancers, has also been found to downregulate *wnt*-5a gene expression (39). This has been more directly addressed in previous interim reports using antisense *wnt*-5a or *wnt*-4 DNA constructs. We have found that antisense *wnt*-4 or *wnt*-5a transfected into C57MG allows for cell transformation to occur. That is, the cells continue to grow at confluence and morphologically become similar to the *wnt*-1 transformed cell phenotype (33). **Therefore, loss of normal *wnt*-4 or *wnt*-5a gene expression in C57MG mouse mammary cells correlates with increased cell proliferation and increased cell saturation density previously reported in the last interim report. In this interim report we are reporting further studies involving *wnt*-5a which we now feel is a candidate tumor suppressor gene.**

Various members of the *wnt*-gene family have been implicated in human tumorigenesis. *Wnt*-2, *wnt*-4, and *wnt*-7b gene expression is aberrantly increased in benign and malignant breast tumors and cell lines (40). Human *wnt*-5a in several malignant

cell lines is also minimally expressed or aberrantly expressed compared to corresponding normal cell lines (41, 42). Although *wnt*-5a gene expression in breast cancer tissue is somewhat elevated, its expression is markedly reduced when compared to corresponding benign tissue (42). That *wnt*-5a expression is elevated in benign tumors including colon adenomas suggests that *wnt*-5a may be functionally related to the early progression of tumorigenesis (41). It is also possible that *wnt*-5a regulates normal cell growth and its expression will transiently increase in transformed cells which is reflecting an attempt to re-establish normalcy. It is of interest that human *wnt*-5a in several normal and malignant cell lines aberrantly express without gene amplification or rearrangement compared to corresponding normal cell lines (41). It has been suggested that the upregulation of *wnt*-5a gene expression in malignant and benign tissue relates to an attempt to regulate cell migration, and that *wnt*-5a downregulation corresponds with increased cell motility (50) due to loss of cell adhesion (43-45).

The short arm of chromosome 3 (3p) in human cancer

Genetic alterations of chromosomes containing tumor supressor genes are thought to be contributing to the multi-stage progression of malignant tumors (46). These alterations may include nonrandom chromosomal deletions or loss of heterozygosity (LOH) (47). The short arm of chromosome 3 (3p) has a particularly high frequency of deletion or rearrangement in human cancers including small cell lung carcinoma, oral squamous cell carcinoma, cervical carcinoma, breast carcinoma, renal cell carcinoma, and uroepithelial cell carcinoma (48-53).

In human renal cell carcinoma, loss of alleles and cytogenetic aberrations have been well documented. For example, up to 89% of nonpapillary forms of renal cell carcinomas have been shown to have a nonrandom loss of chromosome 3p (58, 59). The loss of heterozygosity determined by restriction fragment length polymorphism (RFLP) analysis occurs consistently in renal cell carcinoma suggesting the loss of one or more tumor suppressor genes which likely play a significant role in renal cell carcinogenesis (53). Although the precise location of the 3p tumor suppressor gene(s) is not known, cytogenetic analysis suggests that the region 3p11-3p25 likely carries one or more suppressor genes (53, 56, 57). More specifically, Yamakawa et al. mapped one suppressor gene to 3p13-p14.2 and another distal to 3p21.3 in renal cell carcinoma (58). Another region encompassing 3p12-p14 has been found to dramatically alter tumor growth in nude mice when a fragment containing this region was introduced into a highly malignant nonpapillary renal cell carcinoma cell line (59). This region includes the translocation breakpoint in familial renal cell carcinoma (52, 60, 61). A nontumorigenic human renal cell carcinoma cell line (RCC23) has been established from a stage III nonpapillary carcinoma with a loss of heterozygosity (LOH) on 3p (62). Chromosome and RFLP analysis revealed an unbalanced translocation between chromosome 3p and 8q (t(3;8)(p11;q11)) resulting in the loss of the 3p11-pter region (62). Using RCC23 cells, previous studies showed that microcell hybrids containing an introduced intact chromosome 3 resulted in a significant reduction in growth rate, saturation density, and altered morphologic phenotype compared to the parental cancer cells (62). Furthermore, it was later demonstrated that the introduction of chromosome 3 also restored cellular senescence which was associated with repression of telomerase activity (63).

In bladder cancers, a specific correlation between the loss of chromosome 3p and the development of high grade malignancy has recently been found 52). In support of the hypothesis that genes on chromosome 3p act as tumor suppressors, Wu et al. showed that all somatic cell hybrids which were formed between nontumorigenic SV-HUC-1 cells and an isogeneic derivative transitional cell carcinoma cell line (MC-T16) that lost 3p on initial transformation were tumorigenically suppressed (64, 65). Upon reversion, hybrids were subsequently found to have a deletion of chromosome 3p13-p21.2 (66).

Human *wnt*-5a has been cloned and mapped to human chromosome 3p14-3p21 (42, 67). This past year we have been particularly interested in elucidating the role of *wnt*-5a in tumorigenesis. Preliminary studies reported below suggests that *wnt*-5a is a growth regulating gene and possibly a candidate tumor suppressor gene which may be deleted or rearranged during the multi-step development of events which lead to tumorigenesis. **The experiments outlined in this interim report have important implications for understanding basic mechanisms underlying breast tumorigenesis and indicate that the deregulated expression of a *wnt*-5a may be important in the multi-step progression of all types of cancer.**

BODY OF INTERIM REPORT

TASK 1 and TASK 2 have been completed and have led us to pursue the role of *wnt*-5a in tumorigenesis. This directly relates to our findings that loss of endogenous *wnt*-5a gene expression mimics a *wnt*-1 transformed phenotype. This is also partially based upon recent reports in the literature that *wnt*-5a and *wnt*-4 are differentially regulated in human breast cancer and in other human malignancies which were to be the subject of TASK 3 AND TASK 4. We elected not to repeat published data, and sought instead to adjust this proposal to determining directly whether *wnt*-5a acts as an anti-oncogene in human cancer as proposed in TASK 4. As reported in our previous interim report, we have also continued to use differential display to uncover potential mechanisms of action by *wnt* genes in cell transformation.

Experimental Methods

The following experiments are designed to test directly the hypothesis that *wnt*-5a is a growth regulating gene which may act as a previously undescribed tumor suppressor gene in human cancer. Briefly, it was proposed to determine whether loss of *wnt*-5a using anti-sense technology allows for cell transformation, and whether the ectopic expression of *wnt*-5a in human malignant cell lines with loss of normal *wnt*-5a gene expression can revert the transformed phenotype. As described, we have made substantial preliminary progress in support of the hypothesis.

1. *To determine the importance of wnt-5a in C57MG growth and differentiation.*

Cell Lines

The mammalian expression vector pRSV was blunt ligated to the mouse *wnt*-5a cDNA full length clone (gift of A. McMahon, Harvard) in the antisense orientation which was confirmed by restriction enzyme mapping. The above mammalian expression vector was co-transfected with pSV2NEO (gift of Dr. J. Papkoff, Sugen, Palo Alto, Ca) into C57MG mammary epithelial cells using lipofection as described previously (68). Stable cell lines were selected and then maintained in 250 ug/ml G-418 in Dulbecco's modified media supplemented with 5% defined bovine serum and 5% fetal bovine serum. Several clones expressing antisense *wnt*-5a constitutively in C57MG cells were screened and confirmed by Northern blot analysis using mouse *wnt*-5a sense riboprobes hybridized to 20 ug of isolated total RNA from confluent cell cultures. To determine the effect of anti-sense *wnt*-5a on endogenous *wnt*-5a gene expression, poly (A) RNA was isolated and 2 ug loaded onto 1.2% formaldehyde gels. The RNA blots were probed with ^{32}-P labeled *wnt*-5a

antisense riboprobes and the effect on *wnt*-5a determined by densitometry using NIH Image 1.57 software.

Transformed C57MG cells expressing *wnt*-1 under the control of an RSV promoter were maintained in Dulbecco's modified Eagle (DME) medium supplemented with 5% fetal calf serum and 5% bovine calf serum (HyClone), and 250 ug/ml G-418 (Gibco). The cells were transfected with an expression vector for *wnt*-5a ligated in the sense orientation (provided by Dr. A. P. McMahon, Harvard) using lipofection as described. The cells were co-transfected with an expression vector for hygromycin (SV2HYG, kindly provided by Dr. M. Liskay, Oregon Health Sciences University), and resistant colonies selected and expanded into cell lines. RNA was extracted and cells expressing *wnt*-5a was verified by Northern blot analysis using specific hybridization probes for *wnt*-5a and compared to those cells only transfected with SV2HYG.

Cell growth characterization

To determine the cell saturation density, population doubling time, and morphologic phenotype of C57MG/*wnt*-1 cells, C57MG/*wnt*-1/*wnt*-5a cells, and C57MG/antisense *wnt*-5a cells, cells were plated in 12-well dishes at a density of 4×10^4 cells per well. The cells were counted every day for 8 days, and the growth rate and population doubling time determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence at 6 days. Morphology was determined by allowing the cells to grow to confluence and photographed.

RNA isolation

Total cellular RNA was isolated from dishes of confluent cells (69). Twenty µg of RNA was analyzed on a 1.0 % agarose formaldehyde gel followed by transfer to Hybond-N (Amersham) membrane. The membranes were UV cross-linked (Stratagene) and prehybridized at 42°C for 3-6 hours, and then hybridized at 42°C overnight with full length cDNA *wnt*-5a probes labeled by random priming with [^{32}P]dCTP using $1\text{-}2 \times 10^6$ cpm/ml. Membranes were washed at room temperature twice in 2x SSC, 1% SDS, followed by several washes in 0.1x SSC, 0.1% SDS at 55°C. Since endogenous *wnt*-5a expresion is low, it was necessary to select for poly(A)+ RNA using an oligo-d(T) cellulose column. Two ug of each poly(A)+ sample was analyzed on a 1.2% agarose formaldehyde gel and transferred to a Hybond-N (Amersham) membrane. After cross-linking, the membranes were prehybridized for 3-5 h at 60° C and then hybridized overnight at 62°C with ^{32}P labeled riboprobes made with a Riboprobe system II kit from Promega. Blots were washed at room temperature twice in 2X SSC, 1% SDS, followed by several washes in 0.1X SSC, 0.1% SDS at 65°C. The prehybridization and hybridization solutions consisted of 50% formamide, 4x SSPE, 0.2 mg/ml sheared and boiled salmon sperm DNA, 2.5x Denhardt's, and 1% sodium dodecyl sulfate (SDS). The membranes were then be mounted on 8 x 10 film (Kodak) with an intensifying screen and placed at -80°C up to 5 days.

Soft agar assay.

In triplicate, 10^4 cells were plated in 0.35% agar (Noble) suspension using standard media over a previously poured 0.5% agar base in 12-well dishes. G-418 selected C57MG cells were used as a negative control. C57MG cells expressing ectopic *neu* T (c-erbB2) (from Oncogene Science) were used as a positive control. Three clones of C57MG cells expressing antisense *wnt*-5a were plated for comparison. Colony formation was determined daily for two weeks. Media was added to the wells as needed. Three separate soft agar assays were done and the results pooled.

^{3}H-thymidine incorporation.

Cell lines were plated into 5 cm dishes in standard growth media and grown to confluence with media change every two days (14 days). In triplicate, 4 μCi of ^{3}H-thymidine (methyl ^{3}H, 60-90 Ci/mmol, aqueous, ICN) was added to each dish and incubated for 2 hours at 37°C. The cells were washed twice with phosphate buffered saline (PBS) followed by addition of 2 ml ice cold 10% trichloroacetic acid (TCA) and incubated for 30 minutes on ice. Cells were then washed with 10% TCA followed by addition of 2 ml of 0.1N NaOH. The dishes were incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1N HCL. The dishes were carefully scraped and the extract added to 10 ml of scintillation fluid for counting. A fourth dish of cells was grown in parallel to determine total protein and the counts normalized to total protein. The results reflect the summary of three separate experiments.

2. Transfection of BT-483 ductal mammary carcinoma cells missing wnt-5a gene expression with sense wnt-5a.

Full length human *wnt*-5a cDNA (clone T11) (67) is a kind gift of Dr. Renato Iozzo (Jefferson Medical College, Philadelphia, PA). The cDNA was subcloned into pRSV (Dr. Jackie Papkoff, Sugen, Redwood City, CA) and orientation determined by restriction analysis. BT-483 mammary carcinoma cells characterized previously (ATCC) as missing chromosome 3p were cultured in F12 media supplemented with 1% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin. The media was also supplemented with insulin. Cells were grown to 80% confluence before each passage. For gene transfection, passage 20 cells were grown to 50% confluence and the media exchanged for low serum media (Optimem, Gibco). Using 90 μl liposomes (Lipofectin, Gibco) mixed with 10 μg of pRSV*wnt*-5a and/or pSV2neo in a total volume of 150 μl, the cells were transfected overnight at 37°C in 5% CO_2. The media was replaced and the cells grown overnight in media with 1% FBS without the addition of Geneticin (G-418, Gibco). The cells were then selected in media supplemented with 800 ug/ml G-418.

After two attempts, no stably transfected cells carrying *wnt*-5a were selected despite being able to select for resistance to G-418. The following methods will be used if we are successful in obtaining clonal selection.

To determine the growth rates, cell saturation density, population doubling time, and morphologic phenotype of MC-T16/neo cells and MC-T16/*wnt*-5a cells, cells will be plated in 12-well dishes at a density of 4 x 10^4 cells per well. The cells will be counted every two days for seventeen days, and the growth rate and population doubling time determined from the logarithmic part of the growth curve. The saturation density will be determined from the cell number after the cells reached confluence. Morphology will be determined by growing cells to confluence and photographed.

RNA isolation and analysis. Total cellular RNA will be isolated from dishes of confluent cells (69). Twenty μg of RNA will be analyzed on a 1.0 % agarose formaldehyde gel followed by transfer to Hybond-N (Amersham) membrane. The membranes will be UV cross-linked (Stratagene) and prehybridized at 42°C for 3-6 hours, and then hybridized at 42°C overnight with full length cDNA *wnt*-5a probes labeled by random priming with [^{32}P]dCTP using 1-2 x 10^6 cpm/ml. The prehybridization and hybridization solutions will

consist of 50% formamide, 4x SSPE, 0.2 mg/ml sheared and boiled salmon sperm DNA, 2.5x Denhardt's, and 1% sodium dodecyl sulfate (SDS). Membranes will be washed at room temperature twice in 2x SSC, 1% SDS, followed by several washes in 0.1x SSC, 0.1% SDS at 55^{o}C. The membranes will then be mounted on 8 x 10 film (Kodak) with an intensifying screen and placed at -80^{o}C up to 5 days.

^{3}H-thymidine incorporation. Cell lines will be plated into 5 cm dishes in standard growth media and grown to confluence with media change every two days (14 days). In triplicate, 4 µCi of ^{3}H-thymidine (methyl ^{3}H, 60-90 Ci/mmol, aqueous, ICN) will be added to each dish and incubated for 2 hours at 37^{o}C. The cells will be washed twice with phosphate buffered saline (PBS) followed by addition of 2 ml ice cold 10% trichloroacetic acid (TCA) and incubated for 30 minutes on ice. Cells will be then washed with 10% TCA followed by addition of 2 ml of 0.1N NaOH. The dishes will be incubated at 37^{o}C for 30 min and then neutralized with 0.2 ml of 1N HCL. The dishes will be carefully scraped and the extract added to 10 ml of scintillation fluid for counting. A fourth dish of cells will grown in parallel to determine total protein by the Lowery method and the counts normalized to total protein. The results will reflect the summary of three separate experiments.

Soft agar assay. In triplicate, 10^{4} cells will be plated in 0.35% agar (Noble) suspension using standard media over a previously poured 0.5% agar base in 12-well dishes. A G-418 selected BT-483/neo clone which does not grow in soft agar will be used as a negative control. G-418 resistant MC-T16/neo cells will be used as a positive control. Two clones of BT-483/*wnt*-5a will be plated for comparison. Colony formation will be determined daily for two weeks. Media will be added to the wells as needed. Three separate soft agar assays will be done as above and the results pooled.

Athymic nude mice. The tumorigenic potentials of G-418 selected positive BT-483/neo cells, negative control SV-HUC-1/neo cells and two clones of BT-483/*wnt*-5a will be tested by inoculations into 4-6 week old female athymic nude mice (Charles River). Mice will be housed in sterile bubbles in a temperature and humidity controlled room. Inoculations of 2 x 10^{6} cells/site in a total volume of 0.1 ml will be made s.c. in the right dorsal quadrant as previously described (70). The animals will be examined weekly. Tumors will be removed at six months or when necrotic or when 1.5-2 cm in diameter. Representative sections of tumors will be fixed in formalin for histologic preparation and stained with H and E. Fixed and stained tumors will be examined blindly by three pathologists. Representative pieces of some tumors will be used to initiate tumor cell lines using an explant technique. Tumor tissue will be cut cut into 1 mm^{2} explant fragments after washing in PBS which were then plated onto tissue culture grade dishes in F-12 1% fetal bovine serum supplemented media without G-418. After 1 week, the explants will be grown in the presence of 250 µg/ml G-418 and the cells expanded for later analysis.

Telomerase assay. Two different BT-483/neo cell lines will be compared for telomerase activity to two different BT-483/*wnt*-5a cell lines. Subconfluent cultures will be used to prepare the detergent 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) extracts (71). Telomerase enzyme activity will be measured by using a PCR-based telomeric repeat amplification protocol (TRAP) kit from Oncor, Inc. as per manufacturer's instructions. Each reaction product will be amplified in the presence of an internal TRAP assay standard (ITAS, 36 bp). The TRAP reaction products will be separated by 10% PAGE, dried, and autoradiographed. The basal levels of telomerase activity (ladder formation) will be measured by serial dilution of the protein extracts, and an

appropriate range of protein concentration selected that produces a linear response as described (71). Each set of TRAP assay will include control reaction tubes without any extract, extracts treated with RNase A (200 µg/ml), and positive assay control TSR8 template (2 µl/tube). To quantitate the levels of telomerase activity, the average optical density of first six TRAP bands after primer band will be used as a ratio to ITAS band.

Telomere length. Southern blot analysis of telomere length will be done on and BT-483 cells and on two clones of BT-483 cells transfected and expressing h*wnt*-5a. DNA will be extracted from primary cultured cells at 10-15 population doublings, HinfI digested and 5 ug loaded and separated by electrophoresis on a 0.7% agarose gel. This will be transferred to a nylon membrane, and hybridized to the ^{32}P-labeled telomeric probe $(TTAGGG)_4$ in 6X SSPE-1% SDS at 50°C. Membrane washings will be in 6X SSC-0.1% SDS at 50°C. The mean length of the terminal restriction fragment (TRF) will be determined by densitometry.

3. *Transfection of RCC23 renal carcinoma cells missing wnt-5a gene expression with sense wnt-5a.*

Transfection and cell characterization of RCC23 renal carcinoma cells. Full length human *wnt*-5a cDNA (clone T11) (67) is a kind gift of Dr. Renato Iozzo (Jefferson Medical College, Philadelphia, PA). The cDNA was subcloned into pRSV (Dr. Jackie Papkoff, Sugen, Redwood City, CA) and orientation determined by restriction analysis. RCC23 renal cell carcinoma cells (a gift from Mitsuo Oshimura, Trottoria, Japan) characterized previously (64) was cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin. Cells will be grown to 80% confluence before each passage. For gene transfection, passage 20 cells were grown to 50% confluence and the media exchanged for low serum media (Optimem, Gibco). Using 90 µl liposomes (Lipofectin, Gibco) mixed with 10 µg of pRSV*wnt*-5a and/or pSV2neo in a total volume of 150 µl, the cells were transfected overnight at 37°C in 5% CO_2.The media was replaced and the cells grown overnight in media with 1% FBS without the addition of Geneticin (G-418, Gibco). The cells were then selected in media supplemented with 800 ug/ml G-418. Individual clones were isolated, resistant colonies expanded into cell lines, and maintained in media supplemented with 250 µg/ml G-418 for eventual RNA extraction to determine gene expression of *wnt*-5a.

To determine the cell saturation density, population doubling time, and morphologic phenotype of RCC23/neo cells and RCC23/*wnt*-5a cells, cells were plated in 12-well dishes at a density of 4×10^4 cells per well. The cells were counted every day for 10 days, and the growth rate and population doubling time determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence at 10 days. Morphology was determined by allowing the cells to grow to confluence and photographed.

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Telomere length. Southern blot analysis of telomere length was done on RCC23 and and on two clones of RCC23 transfected and expressing h*wnt*-5a. DNA was extracted from primary cultured cells at 10-15 population doublings, HinfI digested and 5 ug loaded and separated by electrophoresis on a 0.7% agarose gel. This was transferred to a nylon membrane, and hybridized to the ^{32}P-labeled telomeric probe $(TTAGGG)_4$ in 6X SSPE-1% SDS at 50°C. Membrane washings were in 6X SSC-0.1% SDS at 50°C. The mean length of the terminal restriction fragment (TRF) was determined by densitometry.

4. ***Transfection of MC-T16 uroepithelial cancer cells missing wnt-5a with sense wnt-5a.***

Full length human *wnt*-5a cDNA (clone T11) (67) is a kind gift of Dr. Renato Iozzo (Jefferson Medical College, Philadelphia, PA). The cDNA was subcloned into pRSV (Dr. Jackie Papkoff, Sugen, Redwood City, CA) and orientation determined by restriction analysis. SV-HUC-1 (72) and MC-T16 (64) uroepithelial carcinoma cells characterized previously (a gift of Dr. Catherine Reznikoff, University of Wisconsin, Madison) were cultured in F12 media supplemented with 1% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin. The media was also supplemented with essential amino acids, ferritin, dexamethasone, insulin, and glutamine as previously described. Cells were grown

to 80% confluence before each passage. For gene transfection, passage 20 cells were grown to 50% confluence and the media exchanged for low serum media (Optimem, Gibco). Using 90 µl liposomes (Lipofectin, Gibco) mixed with 10 µg of pRSV*wnt*-5a and/or pSV2neo in a total volume of 150 µl, the cells were transfected overnight at 37°C in 5% CO_2. The media was replaced and the cells grown overnight in media with 1% FBS without the addition of Geneticin (G-418, Gibco). The cells were then selected in media supplemented with 800 ug/ml G-418. Individual clones were isolated, resistant colonies expanded into cell lines, and maintained in media supplemented with 250 µg/ml G-418 for eventual RNA extraction to determine gene expression of *wnt*-5a.

To determine the growth rates, cell saturation density, population doubling time, and morphologic phenotype of MC-T16/neo cells and MC-T16/*wnt*-5a cells, cells were plated in 12-well dishes at a density of 4 x 10^4 cells per well. The cells were counted every two days for seventeen days, and the growth rate and population doubling time determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence. Morphology was determined by growing cells to confluence and photographed.

RNA isolation and analysis. Total cellular RNA was isolated from dishes of confluent cells (69). Twenty µg of RNA were analyzed on a 1.0 % agarose formaldehyde gel followed by transfer to Hybond-N (Amersham) membrane. The membranes were UV cross-linked (Stratagene) and prehybridized at 42°C for 3-6 hours, and then hybridized at 42°C overnight with full length cDNA *wnt*-5a probes labeled by random priming with [^{32}P]dCTP using 1-2 x 10^6 cpm/ml. The prehybridization and hybridization solutions consisted of 50% formamide, 4x SSPE, 0.2 mg/ml sheared and boiled salmon sperm DNA, 2.5x Denhardt's, and 1% sodium dodecyl sulfate (SDS). Membranes were washed at room temperature twice in 2x SSC, 1% SDS, followed by several washes in 0.1x SSC, 0.1% SDS at 55°C. The membranes were then be mounted on 8 x 10 film (Kodak) with an intensifying screen and placed at -80°C up to 5 days.

^{3}H-thymidine incorporation. Cell lines were plated into 5 cm dishes in standard growth media and grown to confluence with media change every two days (14 days). In triplicate, 4 µCi of ^{3}H-thymidine (methyl ^{3}H, 60-90 Ci/mmol, aqueous, ICN) were added to each dish and incubated for 2 hours at 37°C. The cells were washed twice with phosphate buffered saline (PBS) followed by addition of 2 ml ice cold 10% trichloroacetic acid (TCA) and incubated for 30 minutes on ice. Cells were then washed with 10% TCA followed by addition of 2 ml of 0.1N NaOH. The dishes were incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1N HCL. The dishes were carefully scraped and the extract added to 10 ml of scintillation fluid for counting. A fourth dish of cells was grown in parallel to determine total protein by the Lowery method and the counts normalized to total protein. The results reflect the summary of three separate experiments.

Soft agar assay. In triplicate, 10^4 cells were plated in 0.35% agar (Noble) suspension using standard media over a previously poured 0.5% agar base in 12-well dishes. A G-418 selected SV-HUC-1/neo clone which does not grow in soft agar was used as a negative control. G-418 resistant MC-T16/neo cells were used as a positive control. Two clones of MC-T16/*wnt*-5a were plated for comparison. Colony formation was determined daily for two weeks. Media was added to the wells as needed. Three separate soft agar assays were done as above and the results pooled.

Athymic nude mice. The tumorigenic potentials of G-418 selected positive control MC-T16/neo cells, negative control SV-HUC-1/neo cells and two clones of MC-T16/*wnt*-5a were tested by inoculations into 4-6 week old female athymic nude mice (Charles River). Mice were housed in sterile bubbles in a temperature and humidity controlled room. Inoculations of 2 x 10^6 cells/site in a total volume of 0.1 ml were made s.c. in the right dorsal quadrant as previously described (70). The animals were then examined weekly. Tumors were removed at six months or when necrotic or when 1.5-2 cm in diameter. Representative sections of tumors were fixed in formalin for histologic preparation and stained with H and E. Fixed and stained tumors were examined blindly by three pathologists. Representative pieces of some tumors were used to initiate tumor cell lines using an explant technique. Tumor tissue were cut cut into 1 mm^2 explant fragments after washing in PBS which were then plated onto tissue culture grade dishes in F-12 1% fetal bovine serum supplemented media without G-418. After 1 week, the explants were grown in the presence of 250 μg/ml G-418 and the cells expanded for later analysis.

Telomerase assay. Two different MC-T16/neo cell lines were compared for telomerase activity to two different MC-T16/*wnt*-5a cell lines. Subconfluent cultures were used to prepare the detergent 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) extracts (71). Telomerase enzyme activity was measured by using a PCR-based telomeric repeat amplification protocol (TRAP) kit from Oncor, Inc. as per manufacturer's instructions. Each reaction product was amplified in the presence of an internal TRAP assay standard (ITAS, 36 bp). The TRAP reaction products were separated by 10% PAGE, dried, and autoradiographed. The basal levels of telomerase activity (ladder formation) were measured by serial dilution of the protein extracts, and an appropriate range of protein concentration selected that produces a linear response as described (71). Each set of TRAP assay included control reaction tubes without any extract, extracts treated with RNase A (200 μg/ml), and positive assay control TSR8 template (2 μl/tube). To quantitate the levels of telomerase activity, the average optical density of first six TRAP bands after primer band was used as a ratio to ITAS band. Two different clones of RCC23 cells ectopically expressing *wnt*-5a were also be compared for telomerase activity as above.

Telomere length. Southern blot analysis of telomere length were done on and MC-T16 cells and on two clones of MC-T16 cells transfected and expressing h*wnt*-5a. DNA was extracted from primary cultured cells at 10-15 population doublings, HinfI digested and 5 ug loaded and separated by electrophoresis on a 0.7% agarose gel. This was transferred to a nylon membrane, and hybridized to the ^{32}P-labeled telomeric probe $(TTAGGG)_4$ in 6X SSPE-1% SDS at 50°C. Membrane washings were in 6X SSC-0.1% SDS at 50°C. The mean length of the terminal restriction fragment (TRF) was determined by densitometry.

Cell migration assays. Initially, MC-T16 cells, SV-HUC-1 cells, and MC-T16/*wnt*-5a cells were grown to confluence in plastic tissue culture dishes. A sterile rubber policeman was used to create the wound in the middle of the dish of confluent cells. The cells were photographed daily until the wounds close. In addition, we used Biocoat Matrigel (Becton-Dickinson) invasion chambers which is useful to study cell invasion of malignant and normal cells. After rehydration of the invasion chambers according to the manufacturer's instructions, MC-T16 cells, SV-HUC-1 cells, and MC-T16 cells ectopically expressing *wnt*-5a were prepared in a suspension in culture medium containing 0.1% BSA at 1 x 10^5 cells/ml for 24 well chambers. The chemoattractant was bFGF, or TNF-alpha at a dosage yet to be determined which was added to the chambers. The cell suspensions (0.5 ml) were added to the chambers. The chambers were then incubated for 18-24 hours at 37°, 5% CO_2. After incubation, the non-invading cells were removed according to the

manufacturer. The cells were stained, and then counted. The data will be presented as the percent invasion through the Matrigel matrix and membrane relative to the migration through the control membrane. The "invasion index" can also be expressed as the ratio of the percent invasion of a test cell over the percent invasion of a control cell.

5. Differential display of C57MG mammary epithelial cells transformed by wnt-1.

C57MG cells were grown to confluence prior to their total cellular RNA extraction (69). To remove chromosomal DNA, 50 ug of total cellular RNA were incubated for 30 min at 37°C with 10 units of human placental ribonuclease inhibitor (BRL, Gaithersburg, MD), 10 units of DNase I (BRL) in 10 mM Tris-Cl, pH 8.3, 50 mM KCL, 1.5 mM MgCl2. After extraction with phenol/chloroform, the RNA was ethanol precipitated and redissolved in RNase-free water. mRNA differential display was done as described previously (73) with the exception that 0.2 ug total RNA was used for reverse transcription in a reaction volume of 20 uL. Oligo-dT degenerate anchored primers for reverse transcription will be T12XA, T12XC, T12XG, and T12XT (synthesized by the molecular biology core facility at the Oregon Health Sciences University), where X is a degenerate mixture of dA, dC, and dG will be used for first strand synthesis. These primers were used in conjunction with a decamer oligonucleotide of arbitrarily defined sequence for subsequent amplification (OPA1-20 from Operon, Biotechnology, Inc., Alameda, CA). In order to minimize errors in the PCR procedure, duplicate samples with the same amount of RNA were done. The cycling parameters were as follows: 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds for 40 cycles followed by 72°C for 5 minutes. The amplified cDNAs were separated on a 6% DNA sequencing gel.

To recover and amplify the differentially displayed cDNA bands of interest.

The DNA sequencing gel was blotted on to a piece of Whatman 3mm paper and dried without fixing. After orientation of the autoradiogram and dried gel, cDNA bands of interest were located. The gel slice along with the 3mm paper was incubated in 100 uL distilled water for 10 minutes. After rehydration of the gel, the cDNA was diffused out by boiling the gel slice for 15 minutes in a microfuge tube. After ethanol recovery, 4 uL of eluted cDNA was reamplified in a 40 uL reaction volume using the same primer set and PCR conditions as used in the mRNA display except the dNTP concentrations were at 20 uM instead of 2-4uM and no isotope was added. 30 uL of PCR samples were run on a 1.5% agarose gel and stained with ethidium bromide. To examine the pattern of gene expression in the sets of cells used for differential display the cDNA bands were removed, amplified, and subcloned into the TA cloning system and the plasmids used for creation of riboprobes after template linearization for Northern analysis. The RNA was isolated as previously described (69) and 20 ug total RNA run on 1.2% RNA gels, and blotted to nylon membranes.

TA cloning of isolated cDNA and sequencing.

Reamplified cDNA was cloned into the pCRII vector using the TA cloning system from Invitrogen (San Diego, CA). Plasmid DNA sequencing of the cloned probes was done with either T7 or SP6 primers using the Sequenase kit from United States Biochemicals Co (Cleveland, OH) (74, 75). The partial sequences obtained were compared to known sequences in data banks. Sequencing reactions were performed in 96-well polycarbonate plates with the use of Sequenase 2.0 Kit (U. S. Biochemical Corp.), S35, dATP, SP6, and T7 primers. Products were run on a 6% polyacrylamide gel made with glycerol tolerant buffer, dried, and exposed to xray film. Gene database searches were performed through the National Center for Biotechnology Information with use of the BLAST network service.

RESULTS AND DISCUSSION

1. Determine whether anti-sense wnt-5a in C57MG mouse mammary epithelial cells leads to cell transformation.

The mammalian expression vector pRSV was blunt ligated to the mouse *wnt*-5a cDNA full length clone (gift of A. McMahon, Harvard) in the antisense orientation which was confirmed by restriction enzyme mapping. The above mammalian expression vector was co-transfected with pSV2NEO (gift of J. Papkoff, Sugen, Palo Alto, Ca) into C57MG mammary epithelial cells using lipofection as described previously (68). Stable cell lines were selected and then maintained in 250 ug/ml G-418 in Dulbecco's modified media supplemented with 5% defined bovine serum and 5% fetal bovine serum. Several clones expressing antisense *wnt*-5a constitutively in C57MG cells were screened and confirmed by Northern blot analysis using mouse *wnt*-5a sense riboprobes to hybridize 20 ug of isolated total RNA from confluent cell cultures. The effect of antisense *wnt*-5a ectopically expression on endogenous *wnt*-5a and RNA levels were done as shown in Figure 1.

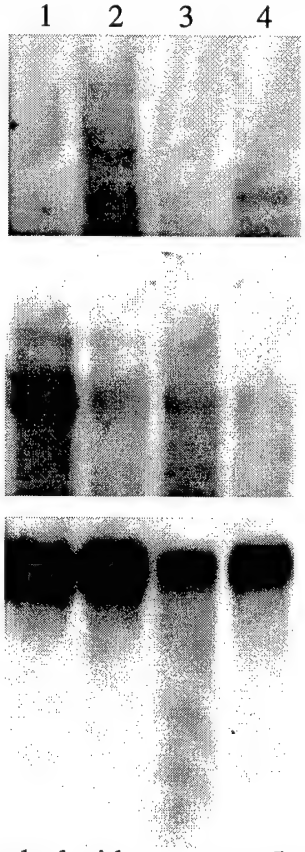


Fig 1. Northern blot probed with sense *wnt*-5a riboprobe for antisense *wnt*-5a expression in top panel compared to the same blot probed with antisense *wnt*-5a riboprobe to determine endogenous *wnt*-5a in middle panel. Lower panel is the same blot probed with actin for RNA loading. Lane 1 is control C57MG cells while lanes 2-4 are three different clones of C57MG cells expressing antisense *wnt*-5a.

A morphology assay was then done by plating 10^5 cells/plate of parental C57MGs, *wnt*-1 expressing C57MGs (known to downregulate *wnt*-5a), and C57MGs expressing antisense*wnt*-5a and a photograph taken at confluence as shown in Figure 2 below.

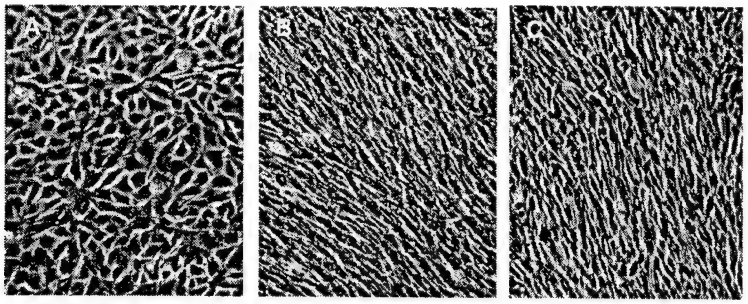
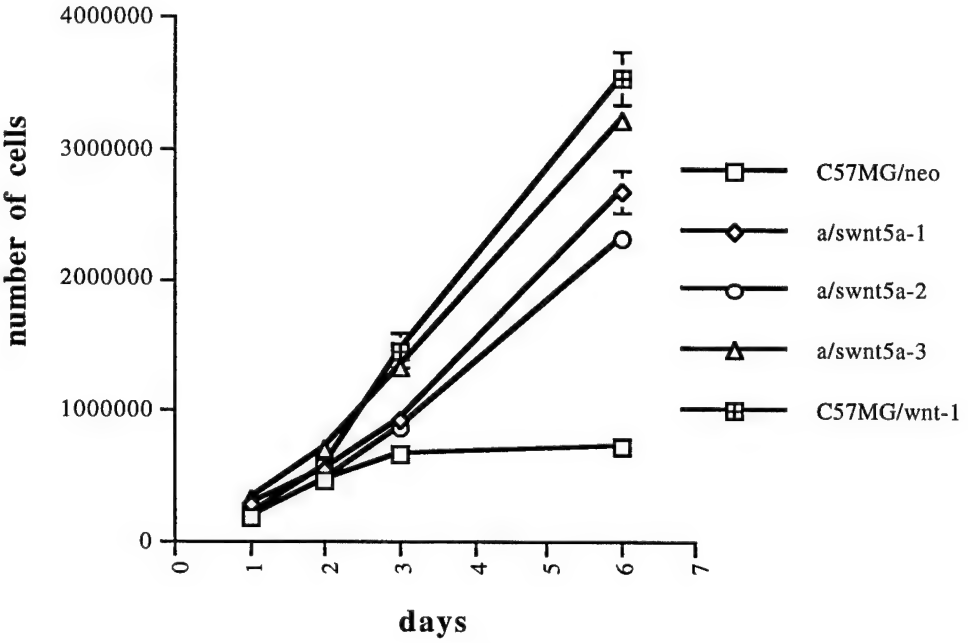


Fig. 2. Photomicrographs of C57MG cells (A) at confluence compared to C57MG cells expressing *wnt*-1 (B) and C57MG cells expressing anti-sense *wnt*-5a (C).

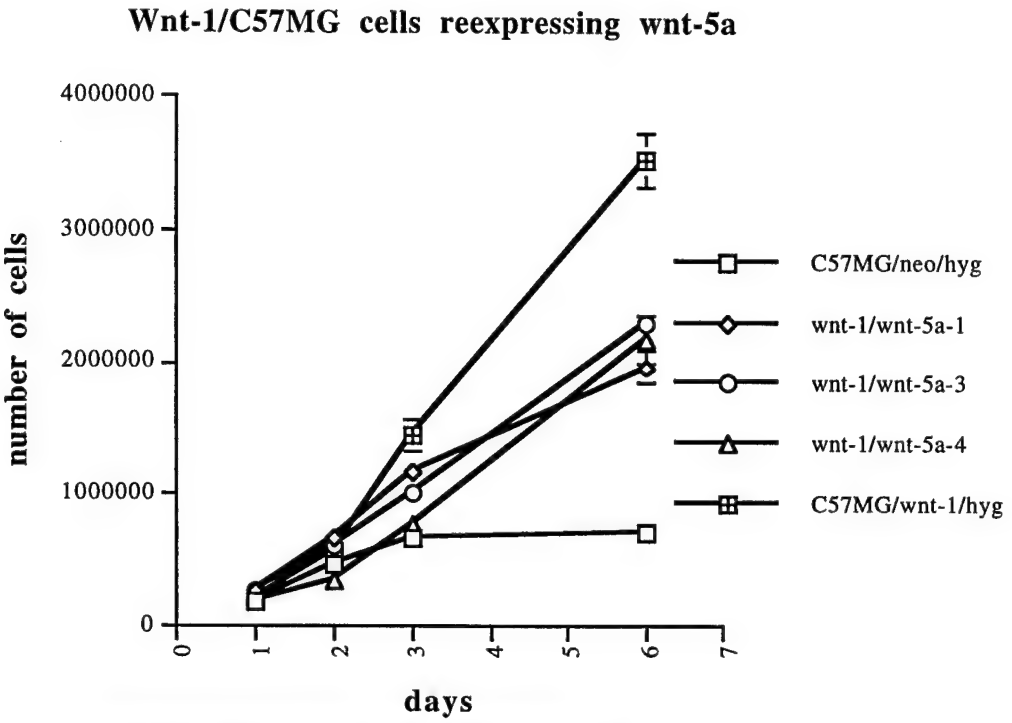
Several clones expressing antisense *wnt*-5a were remarkably similar to the transformed cell phenotype mediated by the ectopic expression of *wnt*-1 (which downregulates endogenous *wnt*-5a in this cell line). Growth curves were done by plating 4 x 10^4 cells and cell counts taken every day for nine days (Figure 3). C57MG cells expressing antisense *wnt*-5a had growth characteristics similar to that found in *wnt*-1 transformed cells (33). ^{3}H-thymidine incorporation was consistent with the presence of antisense *wnt*-5a allowing for loss of normal cell growth and differentiation (data not shown).

C57MG cells expressing antisense wnt-5a



Transformed C57MG cells expressing *wnt*-1 under the control of an RSV promoter were maintained in Dulbecco's modified Eagle (DME) medium supplemented with 5% fetal calf serum and 5% bovine calf serum (HyClone), and 250 ug/ml G-418 (Gibco). The cells were transfected with an expression vector for *wnt*-5a ligated in the sense orientation (provided by Dr. A. P. McMahon, Harvard) using lipofection as described. The cells were co-transfected with an expression vector for hygromycin

(SV2HYG, kindly provided by Dr. M. Liskay, Oregon Health Sciences University), and resistant colonies selected and expanded into cell lines. RNA was extracted and cells expressing *wnt*-5a was verified by Northern blot analysis (data not shown) using specific hybridization probes for *wnt*-5a and compared to those cells only transfected with SV2HYG as shown below.



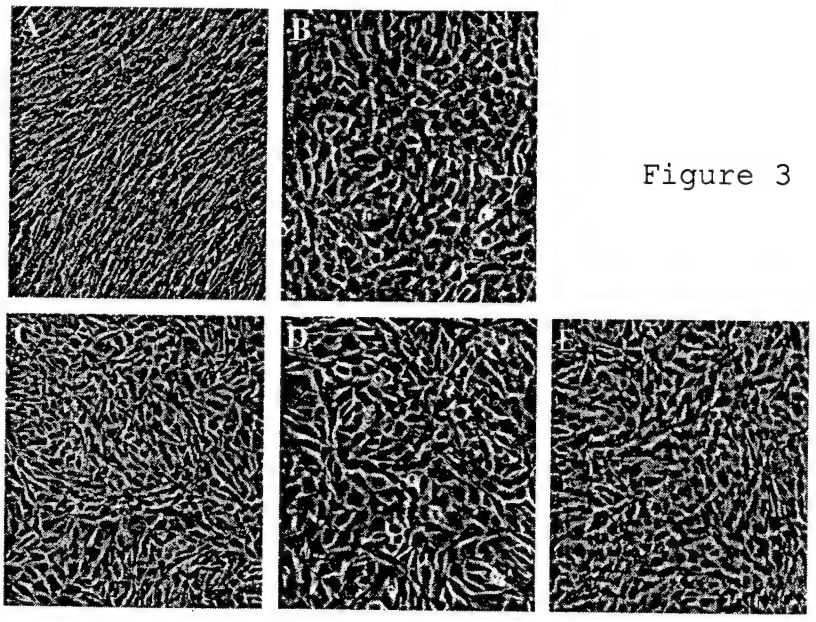


Figure 3

Photomicrographs of three clones stably expressing sense *wnt*-5a in C57MG/*wnt*-1 cells. The parental cells (B) at confluence are compared to transformed C57MG cells expressing *wnt*-1 (A) known to downregulate endogenous *wnt*-5a. Clones expressing ectopic sense *wnt*-5a (C-E) have morphology similar to that seen in the parental cells suggesting that ectopic *wnt*-5a can revert the *wnt*-1 transformed cell phenotype.

2. *Transfection of BT-483 ductal mammary carcinoma cells missing wnt-5a gene expression with sense wnt-5a.*

After two attempts, no stably transfected cells carrying *wnt*-5a were selected despite being able to select for resistance to G-418. The following methods will be used if we are successful in obtaining clonal selection. We elected to try other cell lines known to be missing chromosome 3p as shown below.

3. *Transfection of RCC23 renal carcinoma cells missing wnt-5a gene expression with sense wnt-5a.*

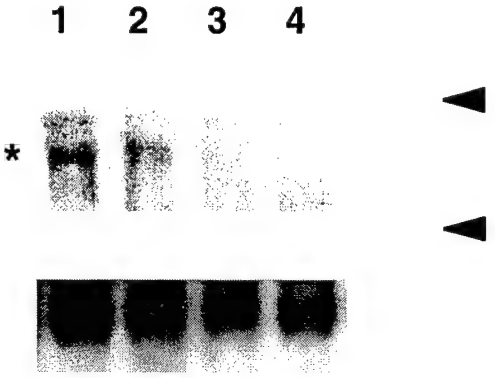


Fig. 4. RNA blot comparing RCC23 renal carcinoma cells (lane 4) to 3 different clones of RCC23 cells expressing ectopic *wnt*-5a (lanes 1-3). The blot was reprobed for actin for RNA loading and integrity.

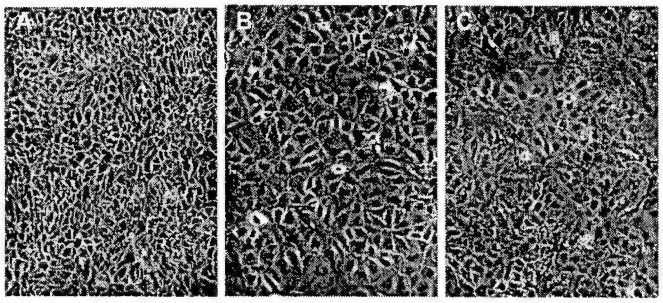


Fig. 5. Photomicrographs of RCC23 renal carcinoma cells which are missing chromosome 3p (A) at confluence compared to two clones of RCC23 cells ectopically expressing *wnt*-5a (B and C).

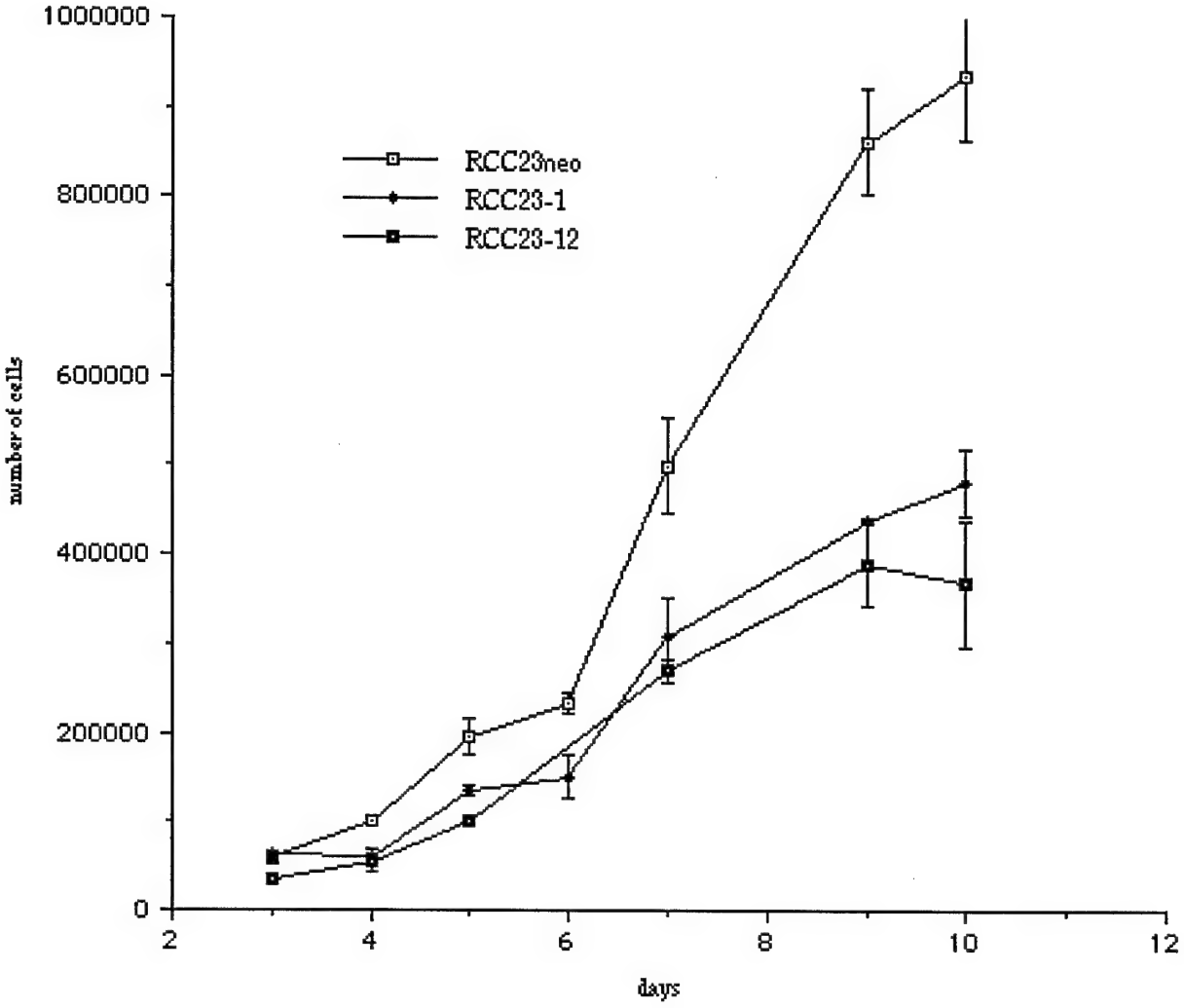


Fig. 6. Growth curves of RCC23 cells compared to two different clones of RCC23 cell ectopically expressing *wnt*-5a.

3. *Transfection of MC-T16 uroepithelial cancer cells missing wnt-5a with sense wnt-5a.*

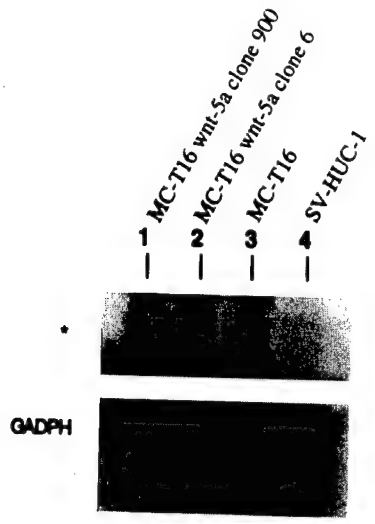


Fig. 7. RNA blot comparing MC-T16 uroepithelial cancer cells expressing ectopic *wnt*-5a (lane 1, clone 900 and lane 2, clone 6) to MC-T16 cells (lane 3) and to parental SV-HUC-1 (lane 4).

Table 1 *Growth characteristics of MC-T16 cells transfected with human wnt-5a.*

Cell type	Generation time (h)	Saturation density (total cells x 10^5)	^{3}H-thymidine incorporation (cpms)	Colony formation (%)
SV-HUC-1	56	3.2 +/- 0.1	8,212	0
MC-T16	26	8.9 +/- 0.8	15,110	9.7 +/- 0.3
MC-T16 h*wnt*-5a-6	32	9.4 +/- 0.7	11,420	0
MC-T16 h*wnt*-5a-900	24	8.0 +/- 0.6	9,435	0.06 +/- 0.03

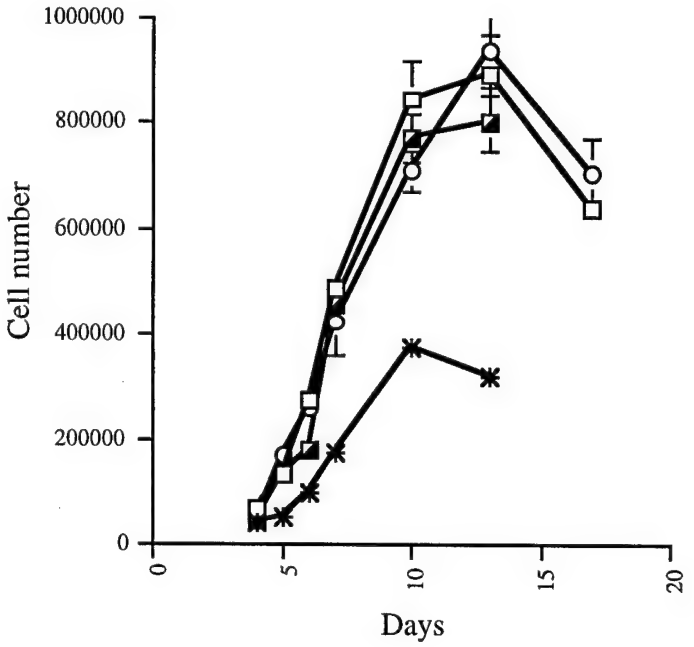


Fig. 8. Growth curves of MC-T16 uroepithelial cancer cells compared to MC-T16 cells ectopically expressing *wnt*-5a and to parental SV-HUC-1 cells (*).

The growth properties were compared between the MC-T16/neo parental cells and two clones (Fig. 7, lanes 1 and 2) of MC-T16/*wnt*-5a to determine whether *wnt*-5a had any influence on growth kinetics. As shown in (Table 1) and (Fig. 8), the growth rate of the MC-T16/neo cells expressing *wnt*-5a was similar to MC-T16 cells in the logarithmic phase unlike that for SV-HUC-1/neo cells and unlike that found in RCC23/*wnt*-5a cells (Fig. 6). That is, the population doubling time of MC-T16/neo cells was 26 hours while that for MC-T16/*wnt*-5a-clone 6 cells was 32 hours and 24 hours for MC-T16/*wnt*-5a-clone 900, in comparison to the doubling time for SV-HUC-1/neo cells which was 56 hours. Cell saturation density at confluence correlated with growth rate. That is, MC-T16/neo parental cells had a saturation density of 8.9×10^5 at confluence, while both MC-T16 clones expressing *wnt*-5a had saturation densities of 9.4×10^5 and 8.0×10^5. This was significantly different than that observed for SV-HUC-1/neo cells which had a saturation density of 3.2×10^5. **Unlike that found for RCC23/*wnt*-5a cell lines, comparing these results suggests that the expression of *wnt*-5a in MC-T16 cells does not alter growth kinetics significantly. This may be due to the immortalization of these cells by SV-40 which is not altered in the presence of *wnt*-5a.**

Morphologic differences in MC-T16/*wnt*-5a cells. The parental SV-HUC-1/neo cells characteristically retain many of the features associated with normal epithelial cells in culture. The isolated SV-HUC-1/neo clone used for these experiments no longer grew at confluence (Fig. 9b) unlike that for SV-HUC-1/neo pooled clones which become tightly packed at confluence (unpublished observations). MC-T16/neo carcinoma cells continue to grow at confluence, are refractive, and have a spindle cell phenotype (Fig. 9a). However, when two different clones of MC-T16/*wnt*-5a cells were examined, the cells at confluence

become less spindle shaped, more flattened, and pleiomorphic (Fig. 9c and d) similar to the SV-HUC-1/neo cells. **These findings suggest that MC-T16 bladder cancer cells ectopically expressing *wnt*-5a are more differentiated than the MC-T16/neo cells.**

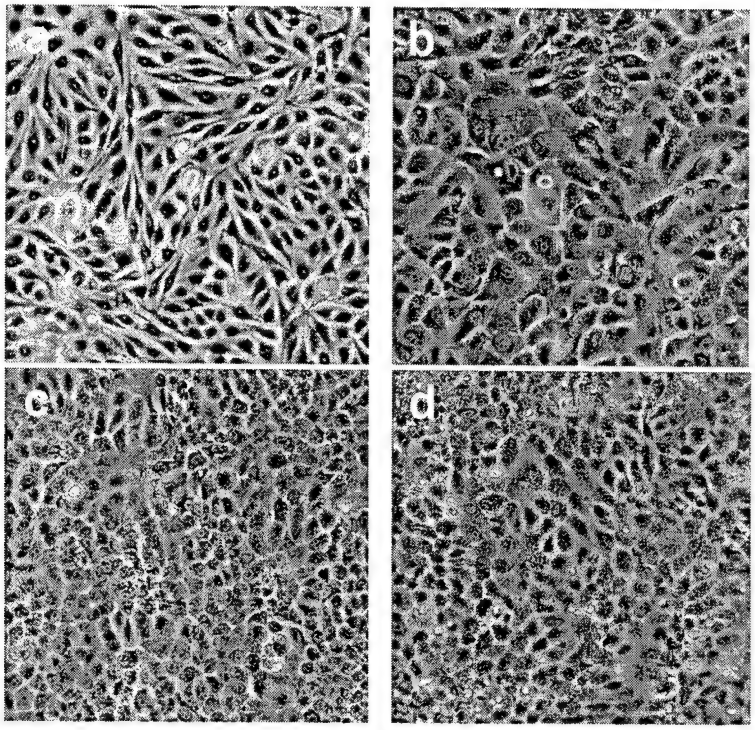


Fig. 9. Photomicrographs of MC-T16 uroepithelial cancer cells (a) compared to parental SV-HUC-1 (b) and to two clones of MC-T16 cells ectopically expressing *wnt*-5a (c, clone 6 and d, clone 900).

Anchorage dependance assay. MC-T16/neo cells grow in 0.35% agar with a cloning efficiency of 9.7% compared to no growth in corresponding parental G-418 selected SV-HUC-1/neo cells (Table 2, below). The expression of *wnt*-5a in MC-T16 cells re-establishes contact dependent growth under these conditions in two different clones. The experiments were repeated three times with similar results.

Table 2. *Soft agar assay comparing cloning efficiencies*

T16 malignant cells	T16-*wnt*5a clone 6	T16-*wnt*5a clone 900	SV-HUC-1 normal cells
9.6%	0%	0.06%	0%

Tumorigenesis in athymic nude mice. Subcutaneous inoculation of MC-T16/neo uroepithelial cells resulted in tumor formation after 4 weeks in 9/10 animals. Spontaneous tumor regression occurred in 4 mice which has been previously described for tumors derived from this cell line (64, 72), and one 1 cm x 1 cm tumor was removed for analysis before the end of the experiment. The remaining tumors grew slowly until the third month when tumor growth rapidly accelerated (Fig. 10). These tumors were removed when greater than 1.5 cm x 1.5 cm or became necrotic. No tumors grew in 10 mice inoculated with SV-HUC-1/neo cells or in 10 mice inoculated with MC-T16/*wnt*-5a-clone 6 cells.

However, 5/5 mice inoculated with MC-T16/*wnt*5a-clone 900 cells grew tumor after a lag time of six weeks. Tumor regression occurred in one animal and one 0.6 cm x 0.8 cm necrotic tumor was removed from another animal. At three months, these tumors grew to a maximum size which never enlarged greater than 1 cm x 1 cm over the following three months (Table 3). The tumors that were examined from mice inoculated with MC-T16/neo cells were attached to underlying tissue and were vascular, while the tumors that grew in mice inoculated with MC-T16/*wnt*-5a-clone 900 cells were found to be unattached to surrounding tissues and remarkably avascular. Tumors from both groups examined histologically confirmed the gross findings. Furthermore, the tumors expressing *wnt*-5a were 80-90% centrally necrotic (Fig. 11a) compared to MC-T16/neo cell derived tumors which were 10-20% necrotic even when considering that the latter tumors grew to a much larger size (Fig. 11b). At higher magnification, little stroma was apparent in MC-T16/*wnt*-5a (Fig. 11c) derived tumors compared to MC-T16/neo tumors (Fig. 11d).

Table 3. *Tumorigenicity of human uroepithelial cells in athymic nude*
Cells at passage 18-20 were grown and 2 x 10^6 cells in 0.1 ml were injected s.c into the right dorsal quadrant of 4-6 week old female athymic nude mice.

Cell lines	Tumor formation	Tumor regression	Tumor size > 1 cm x 1cm
SV-HUC-1	0/10	0/0	0/0
MC-T16	9/10	4/9	4/5
MC-T16 h*wnt*-5a-6	0/10	0/0	0/0
MC-T16 h*wnt*-5a-900	5/5	1/5	0/4

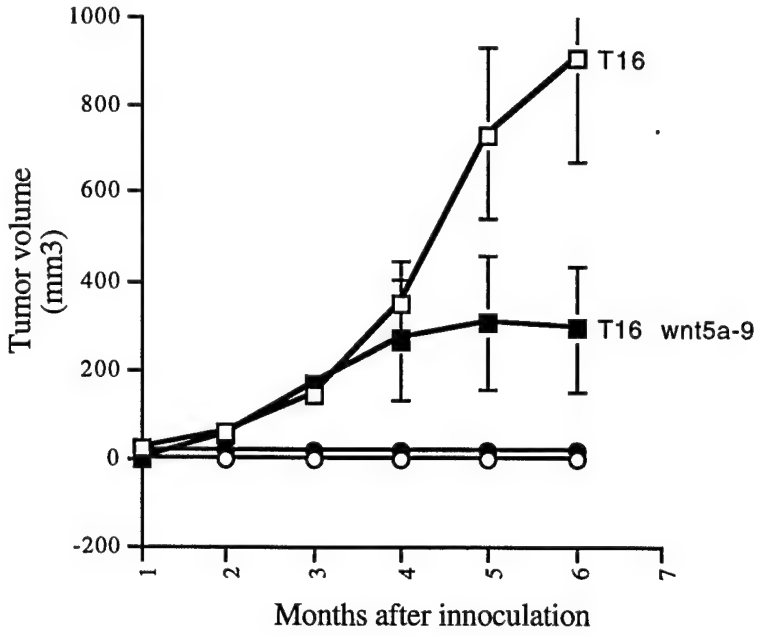


Fig. 10. Tumor volume after inoculation of MC-T16 cells into athymic nude mice compared to MC-T16 cells ectopically expressing *wnt*-5a and to parental SV-HUC- 1 cells. MC-T16/*wnt*-5a, clone 6 and parental SV-HUC-1 tumors did not grow while MCT16/*wnt*-5a clone 900 stopped growing after three months.

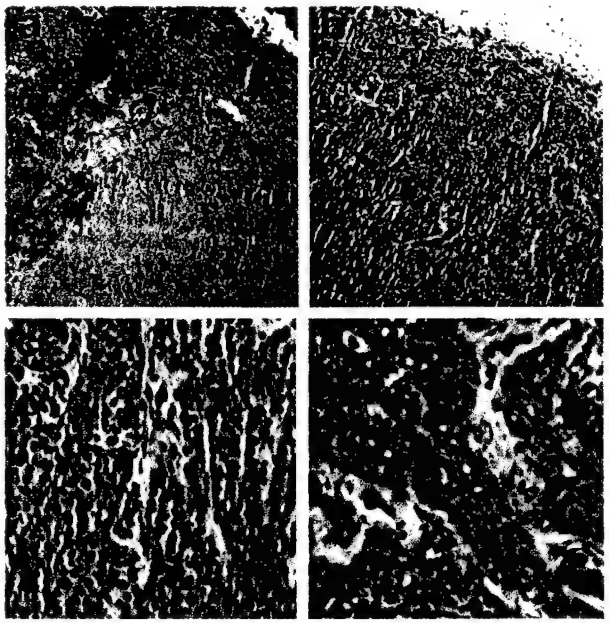


Fig. 11. Photomicrographs of (a) MC-T16/*wnt*-5a derived tumors showing extensive necrosis are compared to (b) MC-T16 uroepithelial cell derived tumors with no necrosis. Higher magnification of MC-T16/*wnt*-5a cells (c) illustrates the lack of stroma when compared to the same magnification of MC-T16 tumors (d) with obvious stroma.

Telomerase assay. Reports have shown that the process of transformation/neoplasia is associated with the activation of telomerase, a ribonucleoprotein enzyme complex that adds telomeric repeats (hexanucleotide 5'-TTAGGG-3') to the ends of replicating chromosomes, or telomeres (76-79), and that another member of the *Wnt* family (25) has been shown to regulate telomerase. We compared telomerase activity between two different G-418 resistant clones of parental MC-T16/neo cells and two clones of MC-T16/*wnt*-5a cells by the primer extension telomere repeat amplification protocol (TRAP) assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers (80, 81). Results indicate no change in telomerase activity in either MC-T16/*wnt*-5a expressing cell lines as shown in Figure.12.

However, **telomerase activity when compared between two different clones of G-418 resistant clones of RCC23 cells to two RCC23 cell lines expressing *wnt*-5a indicate a 5-fold inhibition of telomerase activity** in both RCC23/*wnt*-5a expressing clones as shown in Figure 12, comparing lanes 3, 5, 7, and 9. This discrepancy in the repression of telomerase activity by *wnt*-5a in MC-T16 cells most likely relates to the immortalization of MC-T16 cells by SV-40 which is known to activate teloemerase enzyme activity (71).

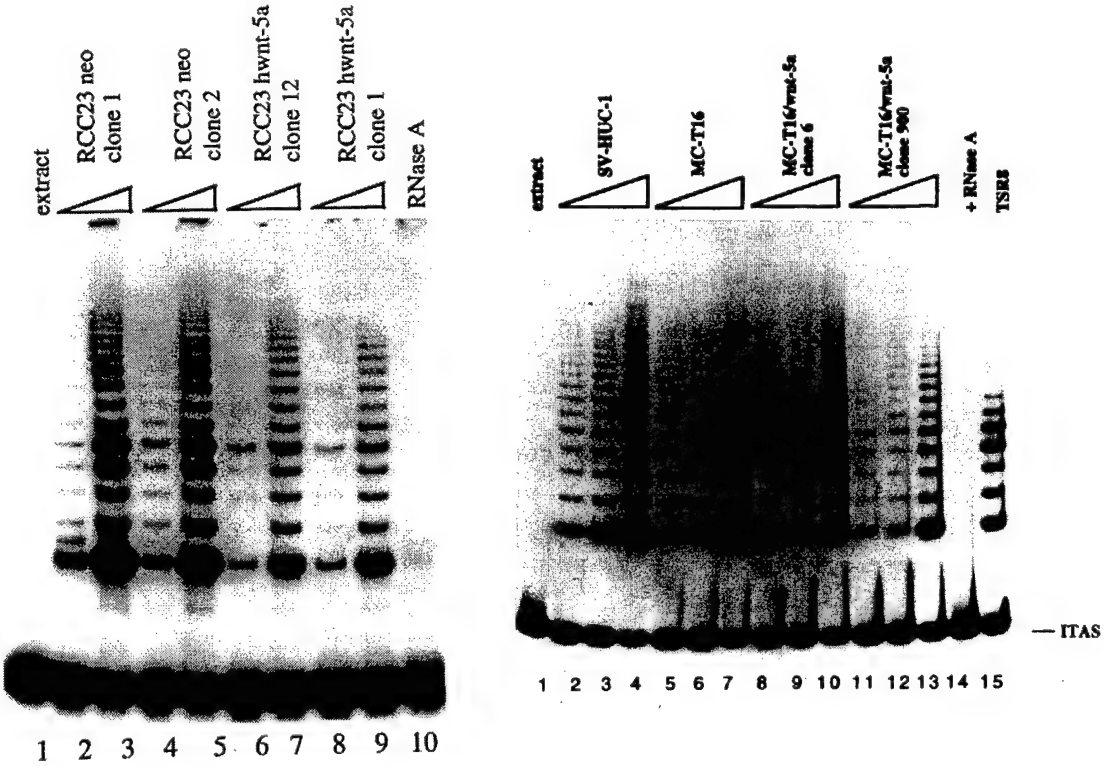


Fig. 12. Telomerase enzyme activity by the primer extension telomere repeat amplification protocol (TRAP) assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers. RCC23 renal cancer cells (A) expressing *wnt*-5a show telomerase repression compared to MC-T16 uroepithelial cancer cells expressing *wnt*-5a (B) which do not show repression.

Determining the whether *wnt*-5a affects cell motility. It has been reported that *wnt*-5a is capable of inhibiting cell motility and migration in two different biologic systems (39, 43, 44). Considering the preliminary findings on reversion of tumorigenesis after inoculation of MC-T16 cells expressing *wnt*-5a *in vivo,* it becomes important to determine in this cell line whether *wnt*-5a alters cell motility. An *in vitro* wound healing assay to ascertain *wnt*-5a effects on cell migration was done using MC-T16 cells compared to SV-HUC-1 parental cells and to MC-T16 cells expressing *wnt*-5a. After 48 hours post-wounding of confluent cell cultures, it was evident that the ectopic expression of *wnt*-5a limits cell motility across wounds as seen in Figure 13. This suggests that one direct effect of ectopic *wnt*-5a in this cell line is to compromise cell migration which ultimately could compromise tumor growth.

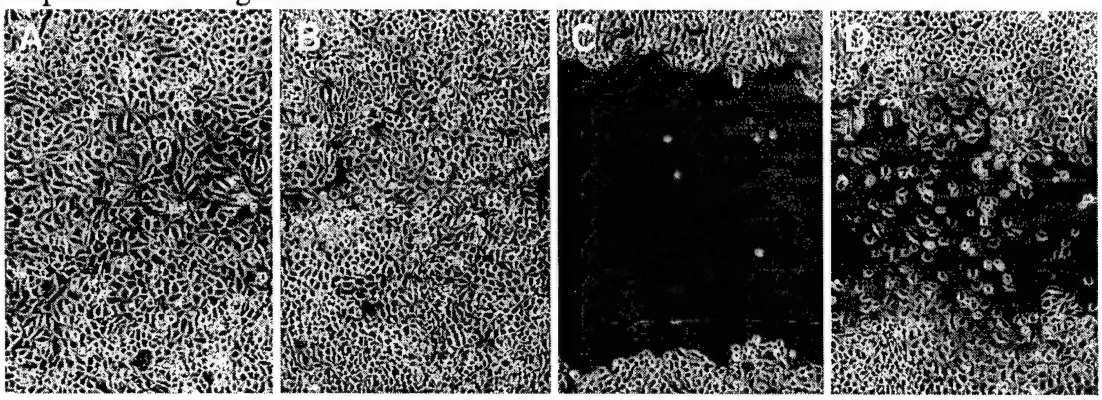


Fig. 13. Photomicrographs of a wound healing assay at 48 hours after wounding confluent cells of SV-HUC-1 (A) compared to MC-T16 uroepithelial cancer cells (B) and to two clones of MC-T16 cells ectopically expressing *wnt*-5a (C, clone 6) and (D, clone 900).

5. *Differential display of C57MG mammary epithelial cells transformed by wnt-1.*

We have previously reported on preliminary results in the 1996 interim report in the isolation and sequencing of one differentially displayed band that had homology to protamine. We have since isolated and sequenced other bands representing differentially expressed genes. Two are of interest and are currently being explored. One has close homology to a intermediate filament-like protein which is upregulated in the presence of *wnt*-1 transformation and downregulates by Western blot analysis in the presence of ectopic *wnt*-5a in human cancer cell lines (data not shown). The other has some homology to an APC-binding protein present in Epstein-Barr virus mediated transformation. The findings will be reported in the final report due in 1998.

CONCLUSIONS

In summary,the data indicates that *wnt*-5a in mouse mammary epithelial cells controls normal growth regulation in that disruption of endogenous *wnt*-5a using anti-sense *wnt*-5a transforms cells in a manner similar to that found when *wnt*-1 or *wnt*-2 transforms cells. The notion that *wnt*-5a controls cell growth was further tested in human cancer cell lines missing chromosome 3p in which *wnt*-5a (mapped to chromosome 3p) was transfected. **We have had difficulty in stably transfecting human mammary carcinoma cell lines missing chromosome 3p** and therefore used other known cell missing chromosome 3p and *wnt*-5a gene expression. In RCC23 renal carcinoma cells, the

ectopic expression of *wnt*-5a differentiates the cell phenotype and represses telomerase enzyme activity in a manner similar to that reported in the literature when microcell hybrids with chromosome 3p were characterized. Since RCC23 cells are not tumorigenic, we repeated the same experiments in MC-T16 cells which are tumorigeneic. The ectopic expression of *wnt*-5a in MC-T16 uroepithelial cancer cells re-established contact dependent growth in soft agar and reverted tumor formation of MC-T16 cells inoculated into athymic nude mice. The mechanism of action of *wnt*-5a in reverting cell transformation and tumorigenesis as suggested by the preliminary studies is beginning to be explored as part of this grant proposal. Evidence suggests that *wnt*-5a may be affecting cell motility. It is known that cell motility and cell migration are necessary for tumor stromal formation and angiogenesis (82). The loss of cell migration as a result of *wnt*-5a may ultimately compromise tumorigenesis. This is currently being explored. **The results presented are consistent with the hypothesis that *wnt*-5a is a growth regulating gene the loss of which can lead to transformation and tumorigenesis. The experiments outlined in this interim report have important implications for understanding basic mechanisms underlying mammary tumorigenesis and indicate that the deregulated expression of a *wnt*-5a may be important in the multi-step progression of breast cancer as well as other cancer types.**

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